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Localization of Spirochetes with the Structural Characteristics of *Treponema hyodysenteriae* in the Lesions of Swine Dysentery

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The large intestines of pigs with swine dysentery were examined by phase, light, and electron microscopy at intervals up to 11 days after oral inoculation with mucosal scrapings from infected pigs. Large spirochetes with the structural characteristics of *Treponema hyodysenteriae* were found only in infected pigs and were first observed in small numbers in the lumen of the large intestine 2 days after inoculation. Numerous large spirochetes were present on the luminal surface and in mucosal crypts as lesions developed. Degenerative changes were first observed in the apical portion of epithelial cells in close contact with large spirochetes. These large spirochetes were found intact in goblet cells and epithelial cells in the early stages of the disease and were numerous within degenerating epithelial cells as lesions became more advanced. Invasion beyond the lamina propria was not detected. These observations demonstrated the relationship between pathogenic large spirochetes and the mucosa of the large intestine in a specific disease, swine dysentery.

Spirochetes are common inhabitants of the large intestine of various species, and some attempts have been made to classify them according to location and host relationships (3, 18, 20). They have been associated with enteric lesions in man (6) and diarrhea in dogs (27), but previous studies (9, 10, 20) have suggested that spirochetes are not primary pathogens in humans, dogs, rats, or rhesus monkeys.

The assumption that spirochetes are insignificant as enteric pathogens has been refuted by recent findings in swine (1, 5, 7, 14, 22, 23, 25). The presence of a variety of spirochetal forms has been noted in normal swine (21), and recently large spirochetes classified as type I by Taylor (21) and as *Treponema hyodysenteriae* by Harris et al. (7) have been found to be associated with the colonic mucosa in the specific disease entity, swine dysentery. Improved anaerobic culture techniques have resulted in the isolation of large spirochetes (*T. hyodysenteriae*) from the colons of pigs with swine dysentery (1, 8, 14, 22). There have also been reports of reproduction of swine dysentery in specific pathogen-free and conventional pigs with pure cultures of *T. hyodysenteriae* (1, 5, 7, 14, 22).

The following report is a summary of experiments involving 21 infected and 8 control pigs. The purpose was to determine the relationship between large spirochetes and the sequential development of lesions of swine dysentery.

MATERIALS AND METHODS

Animals. Pigs weighing 11 to 14 kg were obtained from a closed herd originally stocked with caesarean-derived pigs. All animals were housed in isolation units with principals in one unit and controls in another. Feed consisted of a 16% protein ration free of arsenicals, antibiotics, and other drugs additives.

Inoculation. Twenty-one pigs were inoculated orally with 20 ml of material obtained by suspending scrapings from the colonic mucosa of pigs with acute swine dysentery in phosphate-buffered saline, pH 7.4. Eight pigs served as uninoculated controls.

Specimen collection and preparation. Inoculated pigs were killed at days 1 through 9 and day 11 postinoculation (DPI). Controls were killed at 0, 2, 3, 5, 6, 8, and 11 days after the principals were inoculated. Specimens were collected from the cecum and from three sites in the colon for phase, light, and electron microscopy. Fresh mounts of scrapings from the mucosal surface were used for phase microscopy. Adjacent specimens were fixed in 10% buffered Formalin for light microscopy and in 1.4% glutaraldehyde for electron microscopy.

Formalin-fixed tissues from the cecum and colon were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Warthin-Starry stains. Sections from the small intestine and the paren-

chymatous organs were also stained with hematoxylin and eosin.

The tissues for electron microscopy were fixed for 6 to 8 h before they were removed from the glutaraldehyde, cut into 1- to 2-mm blocks, and rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 9.2% sucrose. Tissues were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Sections 1 μm thick were stained with Paragon Multiple Stain (Paragon C. & C. Co. Inc., Bronx, N. Y.), and specific sites were selected with a light microscope to be sectioned for electron microscopy. Thin sections (approximately 50 nm) were cut on an ultramicrotome (LKB 4801A) and stained with 5% uranyl acetate in 50% alcohol and with 0.3% lead citrate before observation with a Hitachi HU-11A electron microscope at 50 kV.

Salmonella isolation procedures. Colon segments from all pigs were opened by aseptic techniques, and contents were streaked on Tergitol-7 agar (Difco). Approximately 1 g of mucosal scrapings was added to tubes containing 20 ml of tetrathionate broth (Difco). Subcultures were made on brilliant green agar (Difco) after 24 h of incubation at 37 C. Lactose-negative colonies were identified as outlined by Oetjen and Harris (13). Samples of the inoculum used in these experiments were also cultured by the same methods.

RESULTS

Phase microscopy. Small, tightly coiled spirochetes approximately 3 to 5 μm in length and 200 to 250 nm in diameter were present in mucosal scrapings and contents from the colons of all pigs in the study; there was no apparent change in numbers in infected pigs. Numbers were usually higher in the lumen than in the mucosa. Vibrio-like organisms were observed in normal and affected animals.

Large spirochetes were loosely coiled, 5 to 8 μm in length, 300 to 400 nm in diameter, and highly motile. They were never seen in control animals and were first observed in the colonic mucosa of infected animals 2 DPI, which was prior to the first observations (3 to 6 DPI) of clinical signs and gross lesions of swine dysentery. Numbers were greatly increased in animals with early clinical signs and lesions.

Light microscopy. The only consistent histological lesions were in the large intestines of infected pigs. A progression from catarrhal to mucofibrinous to fibrinonecrotic enteritis was observed as the disease progressed from early to later stages. The initial mucosal lesions, which were present when the first clinical signs of diarrhea were noted, included hyperemia and edema with exudation of fibrin. Mucus and fibrin accumulated within mucosal crypts and on the luminal surface. There were also increased numbers of mitotic figures in elongated, darkly stained epithelial cells at the base of colonic crypts. As dysentery with blood and mucus in the feces began, the lesions progressed, and groups of intact epithelial cells could be seen to be separated from the edematous lamina propria near the lumen. Goblet cell hyperplasia was pronounced in the early course of the disease, but diminished in later stages.

Numerous large spirochetes were observed at the luminal surface and deep in mucosal crypts of the large intestine wherever early lesions were noted. These organisms were demonstrated in the epithelial layer, often within goblet cells. The Warthin-Starry stain was useful for locating the organisms (Fig. 1). Large spirochetes were present in the lamina propria in more advanced lesions of mucohemorrhagic enteritis. It was apparent that necrosis of the epithelium was not necessary to facilitate invasion, because crypt epithelial cells containing large spirochetes were intact as seen by light microscopy, except for differences in staining intensity in some areas.

Small, straight, or curved rod-shaped bacteria were sometimes present in the mucosal crypts along with the spirochetes, but they were not seen within cells, except in cases where degeneration was quite advanced.

Electron microscopy. A few small spirochetes similar to those observed with phase microscopy were found near the mucosal surface of both infected and control animals. They were approximately 200 to 260 nm in diameter and had two to four axial fibrils.

Large spirochetes were never observed in the large intestines of controls, but were found at the luminal surface of the colons of infected pigs prior to the observation of clinical signs or gross lesions.

When gross and microscopic lesions were first observed, numerous large spirochetes were located near the surface of the colonic epithelial cells or were massed in the crypts (Fig. 2). The spirochetes observed in these sections had a protoplasmic cylinder approximately 300 nm in diameter with an amorphous granular appearance and no organelles. The protoplasmic cylinder was surrounded by an envelope with a trilaminar appearance. The maximal diameter, including the envelope, was approximately 380 nm. The organisms had a loose, spiral appearance, and a bundle of 12 to 16 axial fibrils (10- to 12-nm diameter) was seen spiraling around the organisms between the protoplasmic cylinder and the envelope (Fig. 3).

The most consistent change in epithelial cells adjacent to the spirochetes was alteration of microvilli, which were shortened, irregular, and sparse in contrast to those from control animals.

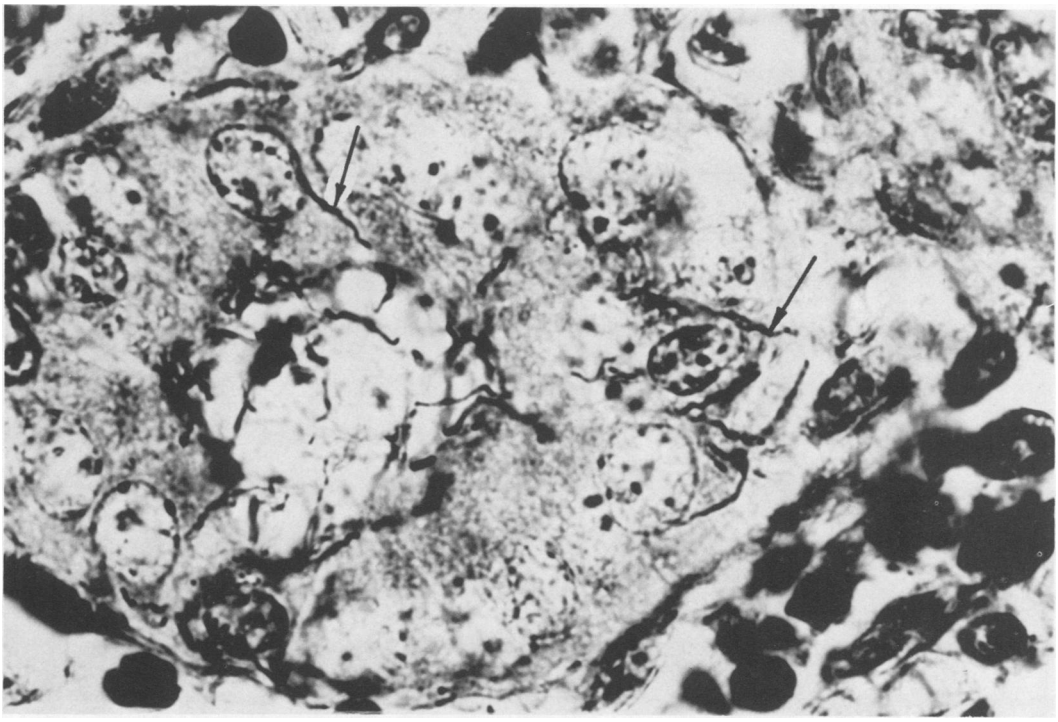

FIG. 1. *Cross-section of a colonic crypt with large spirochetes (arrows) in the lumen and the epithelium. Warthin-Starry stain. ×1,600.*

Mitochondria in some cells were swollen (Fig. 4). There were spirochetes in mucigen droplets and the cytoplasm of goblet cells. The cytoplasm surrounding the mucigen in those cells appeared to be condensed and contained granular material in the endoplasmic reticulum and cytoplasmic matrix. Adjacent epithelial cells were often lightly stained and had decreased numbers of organelles and sparse, irregular microvilli (Fig. 5).

Spirochetes were often found either singly or in clusters within epithelial cells with swollen endoplasmic reticulum (Fig. 6). In most cases they were free in the cytoplasm with no evidence of membranes surrounding them, but occasional clusters of spirochetes were observed within dilated, rough endoplasmic reticula (Fig. 7). The spirochetes within cells were usually intact and appeared not to be damaged by the intracellular environment in normal or degenerate cells (Fig. 8). The intracellular spirochetes were sometimes observed at the base of epithelial cells adjacent to the lamina propria (Fig. 9).

Penetration of the epithelium by spirochetes occurred either between epithelial cells or through the luminal surface as previously reported (5). There was no evidence of specific attachment sites, but invasion seemed to occur by simple invagination of the cell membrane as discussed by Davis et al. (3), who observed spirochetes within the epithelial cells of apparently normal rats.

Bacteriology. Cultures of inoculum and of colons from all pigs at the time of necropsy were negative for *Salmonella* spp.

DISCUSSION

Dubos et al. (4) called attention to the importance of anaerobic bacteria in the large intestine and suggested methods of classifying these organisms according to their relationship with the host. Savage and McAllister (17) stressed the competition between various types of intestinal flora and also noted the specific localization of certain organisms in layers in the mucus on the epithelium of the cecal and colonic mucosa of mice (18). Davis et al. (3) extended these observations to rats. More recently, similar studies were conducted in dogs and rats by Leach et al. (9), who concluded that spirochetes were most numerous in the crypts of the large intestine. When diarrhea was induced in rats with magnesium sulfate, mucosa-associated spiral organisms appeared in large

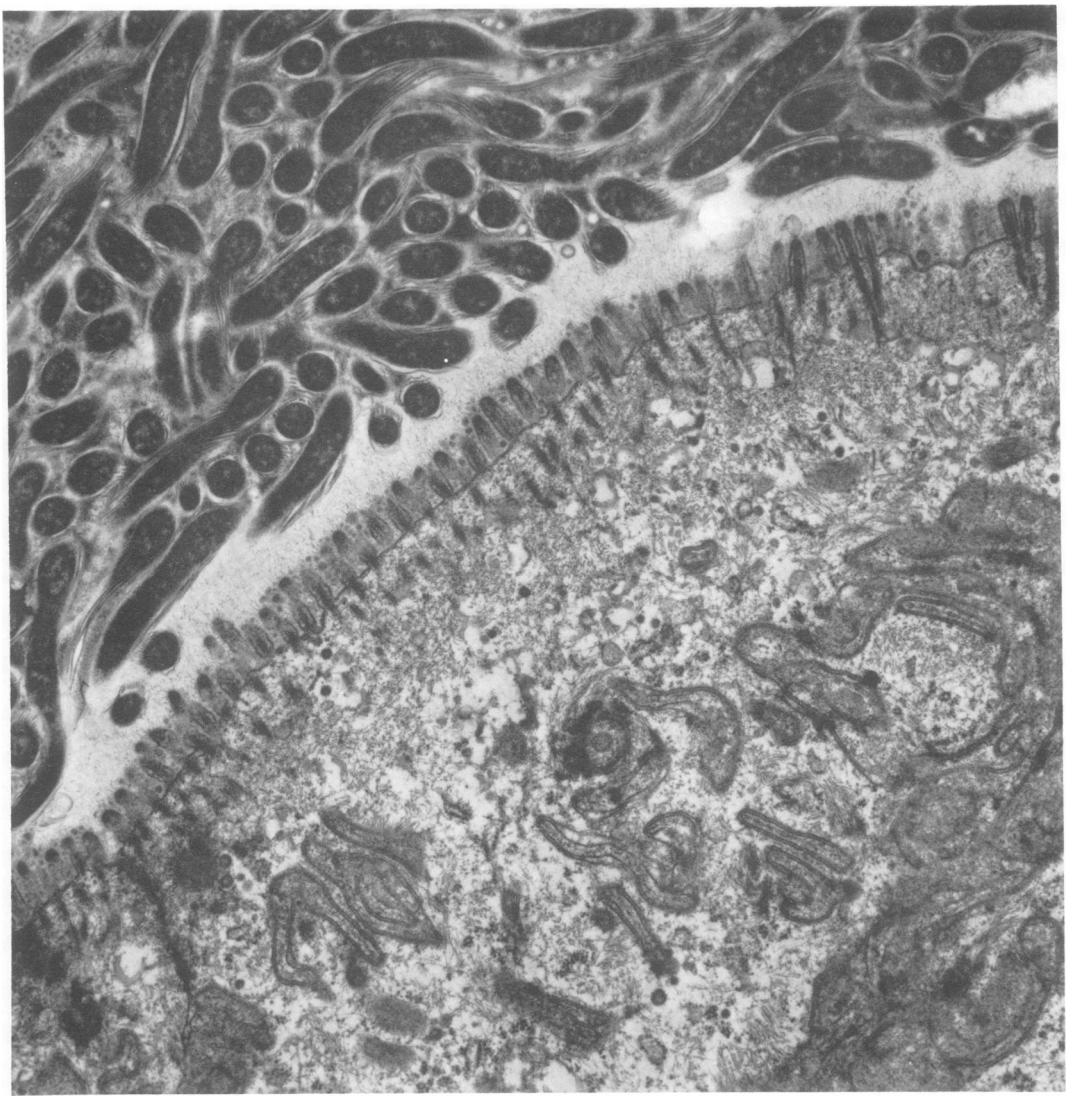

FIG. 2. *Colonic crypt with large spirochetes adjacent to an epithelial cell with short, irregular microvilli.* ×*14,700.*

numbers in the stools. They concluded, on the basis of these findings, that intestinal spirochetosis of various species did not reflect any pathogenicity of these organisms.

Swine apparently are unique in that they are the only species in which pathogenic enteric spirochetes have been demonstrated as described in this paper and previous investigations. Whiting et al. (26) first observed the presence of numerous, large spirochetes in the mucosa of the large intestine of pigs with swine dysentery. Interest in the possible role of spirochetes was renewed when Terpstra et al. (24) demonstrated the frequent presence of large spirochetes in the feces of infected pigs by fluorescent antibody techniques. On the basis of these observations, Taylor and Alexander (22) and Harris et al. (8) independently isolated a large spirochete classified as type I by Taylor and Alexander and as *T. hyodysenteriae* by Harris et al. Oral inoculation of pigs with cultures of these organisms which were morphologically identical to the large spirochetes described in this paper produced a disease with

the clinical signs and lesions of swine dysentery (1, 5, 7, 14, 22).

Infected colonic mucosa rather than pure cultures of *T. hyodysenteriae* were used as inoculum in the studies reported in this paper. This work was conducted before pure culture studies were completed, and culture techniques were efficient enough to permit inoculation of large numbers of animals. These findings are presented to support pure culture studies and to better demonstrate the relationship between pathogenic spirochetes and the intestinal mucosa.

Intestinal spirochetes vary greatly in size, shape, location, and metabolic activity. Because of the difficulty in classifying these organisms specifically, it is more convenient to make generalizations on the basis of structural differences as previously suggested (11). For instance, small, tightly coiled spirochetes which were approximately 3 to 5 μm in length, 200 to 260 nm in diameter, and which had 2 to 4 axial fibrils were commonly observed in this study in the lumen of the large intestine of normal swine. They were present in lower numbers in mucosal scrapings and were not found in significant

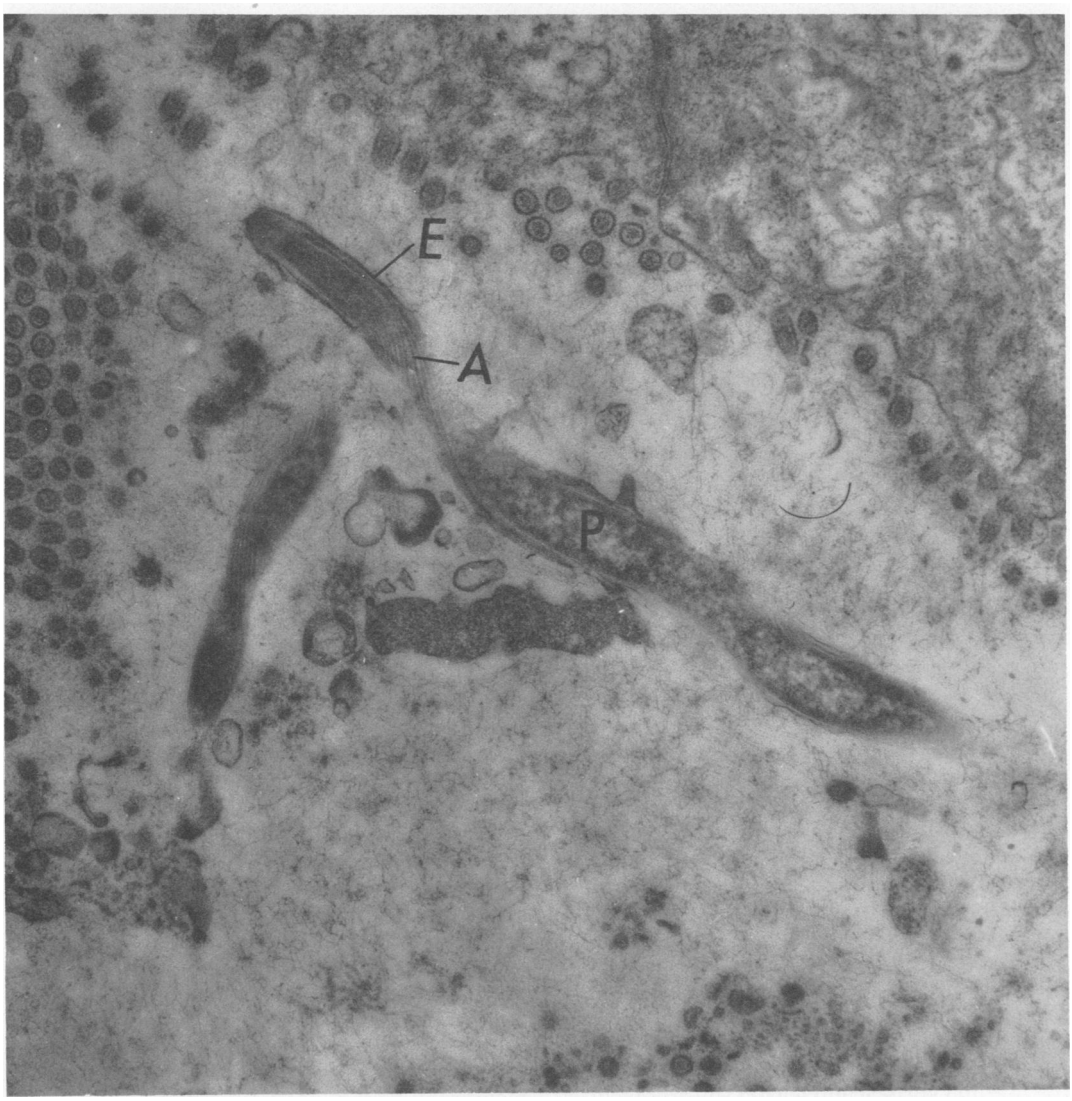

FIG. 3. *Large spirochete in a colonic crypt. The protoplasmic cylinder (P) and axial fibrils (A) are enclosed within the envelope (E).* ×22,200.

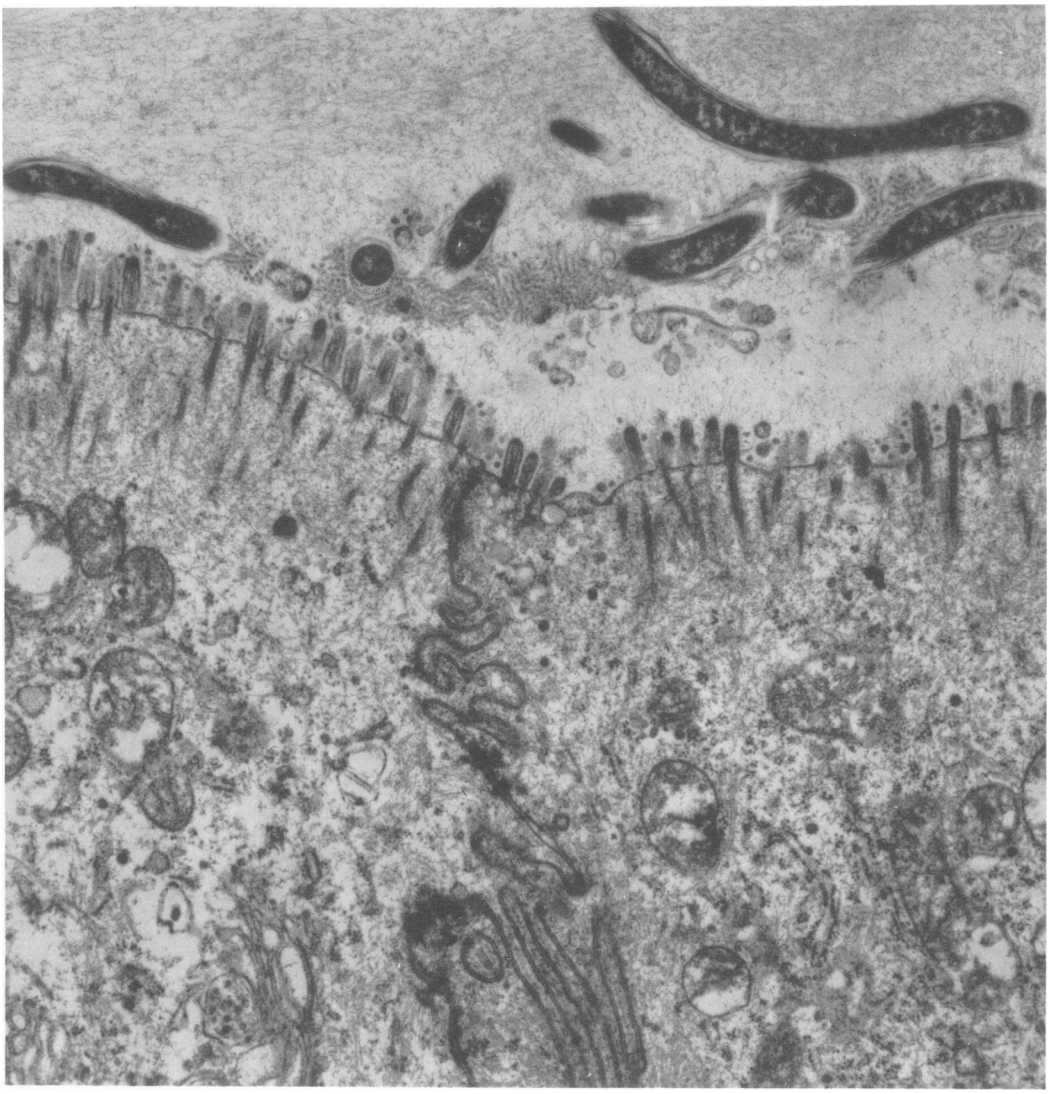

FIG. 4. *Large spirochetes adjacent to colonic epithelial cells which have short, irregular microvilli and swollen mitochondria.* ×*17,500.*

numbers in the mucosal crypts.

In contrast, large spirochetes were not observed in any site in the large intestine of normal swine. However, these spirochetes, which were loosely coiled, 5 to 8 μm in length, 300 to 380 nm in diameter, and which had 12 to 16 axial fibrils, began to appear at the luminal surface prior to the development of lesions of swine dysentery. As lesions developed, numerous large spirochetes were invariably present at the luminal surface and within the crypts, often to the exclusion of other organisms. Adjacent microvilli often were degenerate, and as the disease progressed the large spirochetes could be observed in goblet cells and in and between viable, but damaged, epithelial cells. Specific attachment sites were not demonstrated in this or other studies (5, 23), and it would appear that these highly motile organisms invade cells by a process of invagination of the cell membrane with subsequent engulfment as described by Takeuchi (19) in salmonellosis. However, in no instance were spirochetes observed within a membrane-bound vacuole. They appeared to remain viable and free within the cytoplasm, except when occasionally found to be enclosed

by rough endoplasmic reticulum. There was no evidence of loss of spirochetal envelope membranes. The lesions and spirochete-cell relationships described above correspond closely with previous observations made by Taylor and Blakemore (23) in cases of swine dysentery.

Once the spirochetes have invaded the colonic epithelium, they may continue to multiply within the epithelial cells, which often become contracted and vacuolated. The organisms may also invade the lamina propria, but have not been found beyond this point. Accordingly, the lesions of swine dysentery are limited to the large intestine.

Although there is sufficient evidence to indicate that a specific type of large spirochete classified as *T. hyodysenteriae* is a primary cause of swine dysentery, some other observations should be considered. Harris et al. found that *T. hyodysenteriae* produced enteric lesions after oral inoculation of specific pathogen-free pigs. Germfree pigs were inoculated at the same time and, although the spirochetes were established in the digestive tract, no lesions were produced (D. L. Harris, R. D. Glock, and R. C. Meyer, Abstr. Annu. Meet. Amer. Soc. Microbiol., 72nd, Philadelphia, p. 118, 1972). Dietary factors may have been involved, but it seems

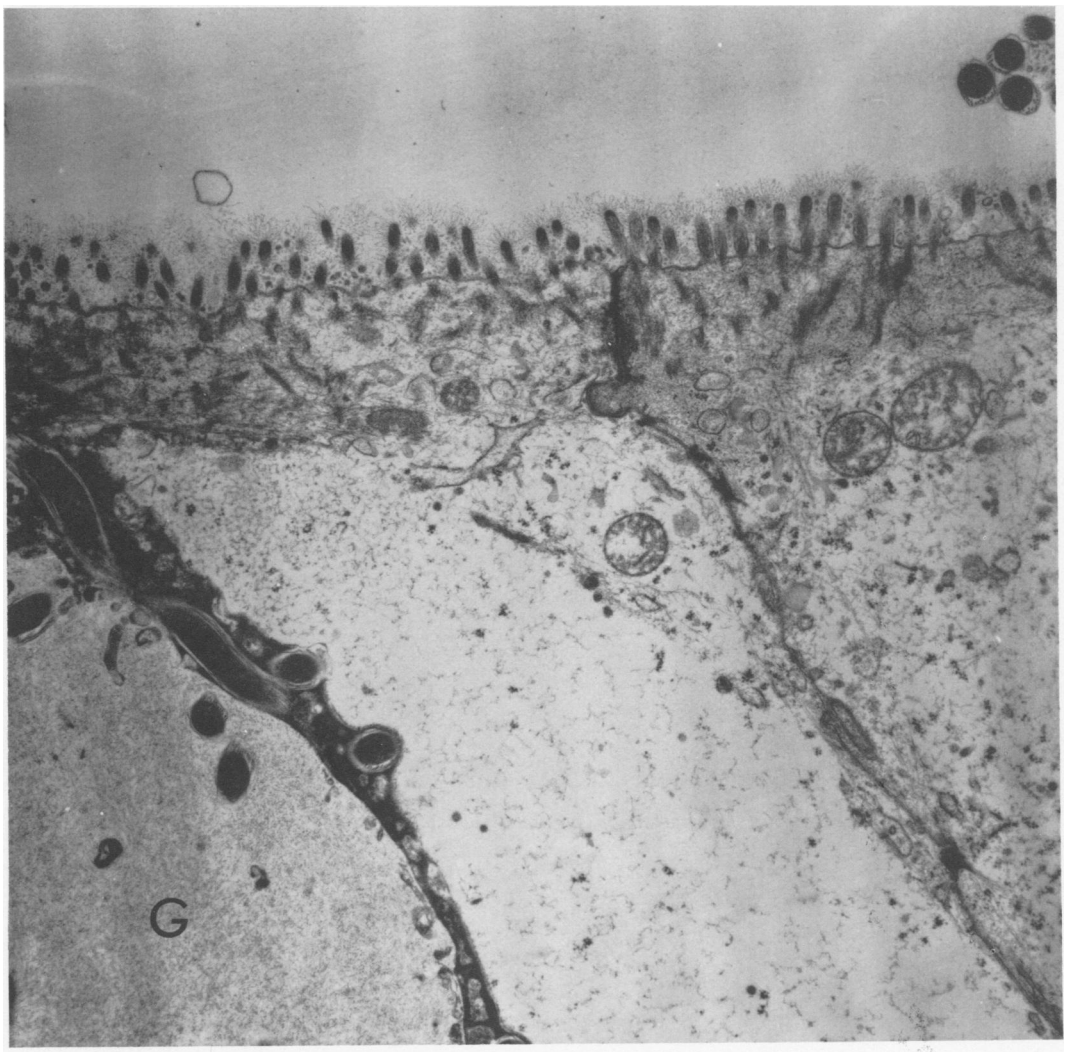

FIG. 5. *Goblet cell (G) with large spirochetes in the mucigen and cytoplasm. The adjacent epithelial cell is lightly stained and has few organelles.* ×15,800.

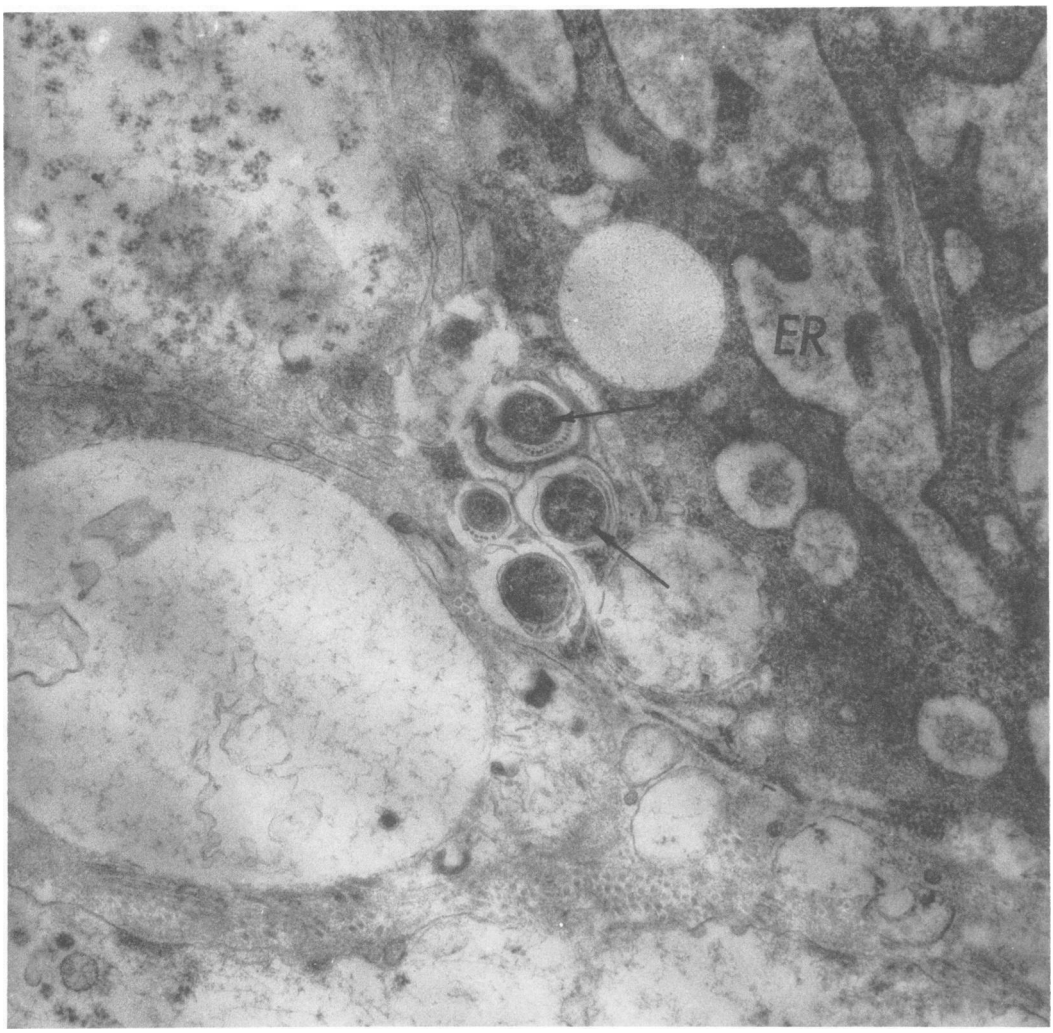

FIG. 6. *Large spirochetes (arrows) within an epithelial cell with swollen endoplasmic reticulum (ER).* ×*31,000.*

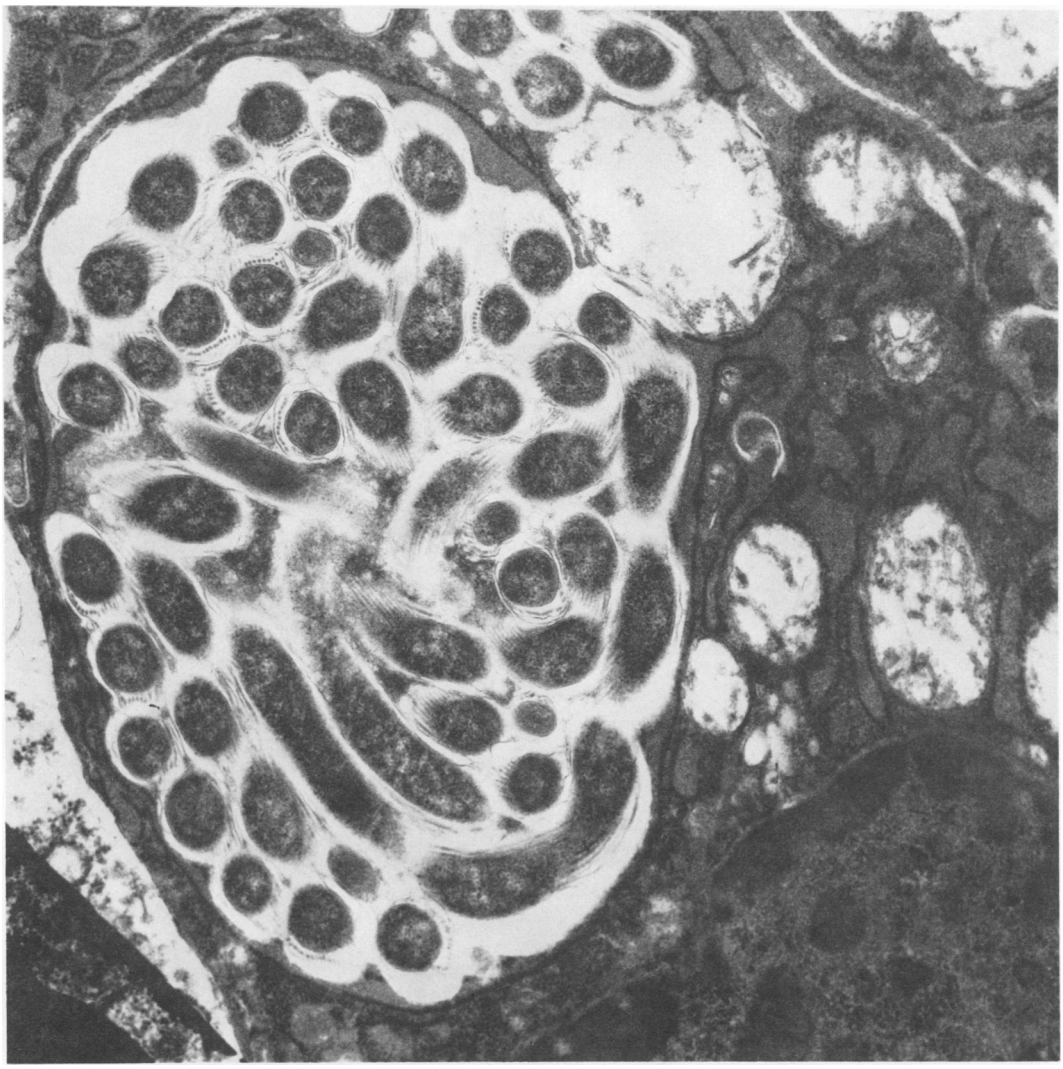

FIG. 7. *Group of large spirochetes within dilated rough endoplasmic reticulum of a colonic epithelial cell.* ×*26,300.*

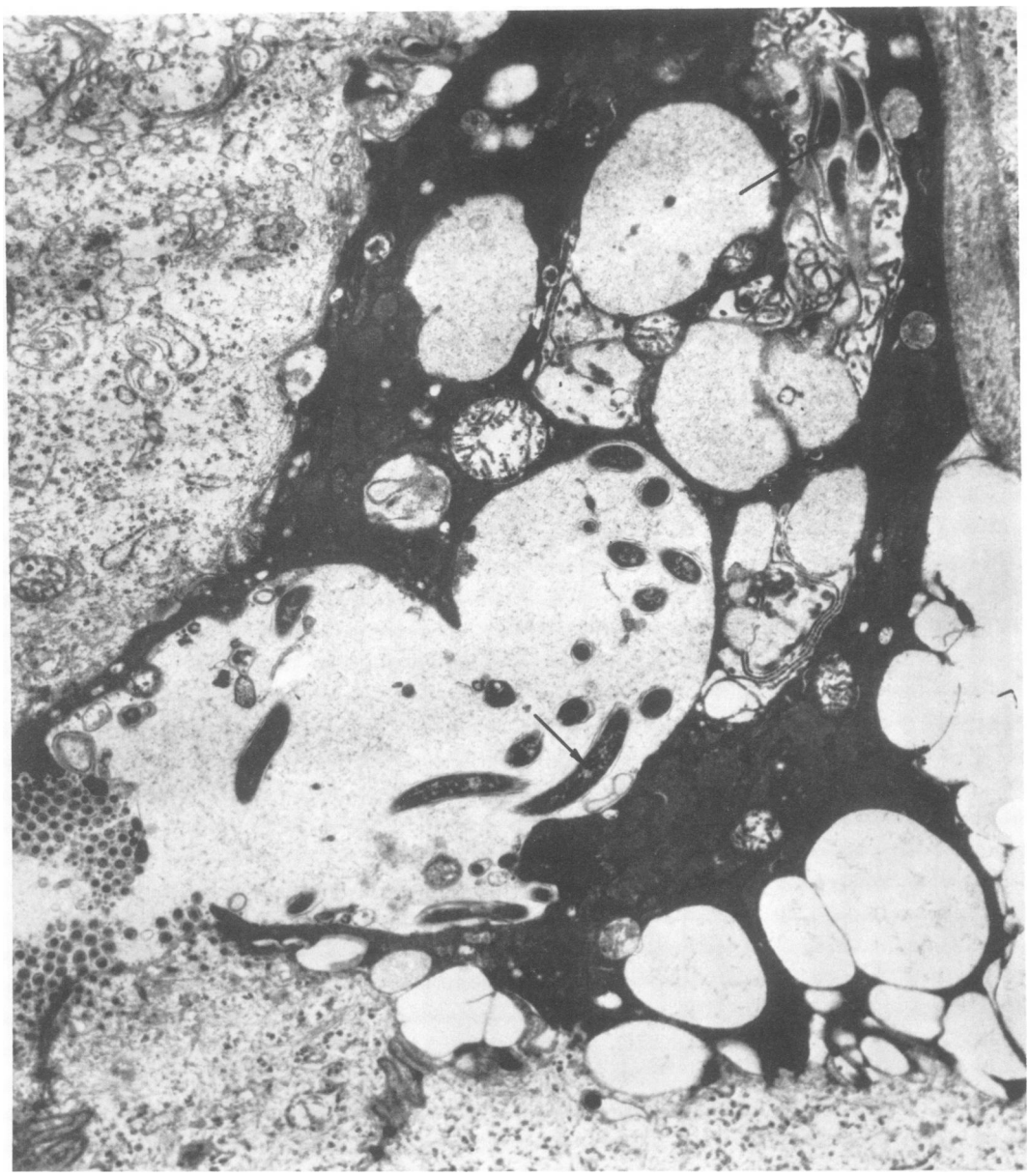

FIG. 8. *Large spirochetes (arrows) within a degenerate, electron-dense cell at the base of a colonic crypt.* ×*11,500.*

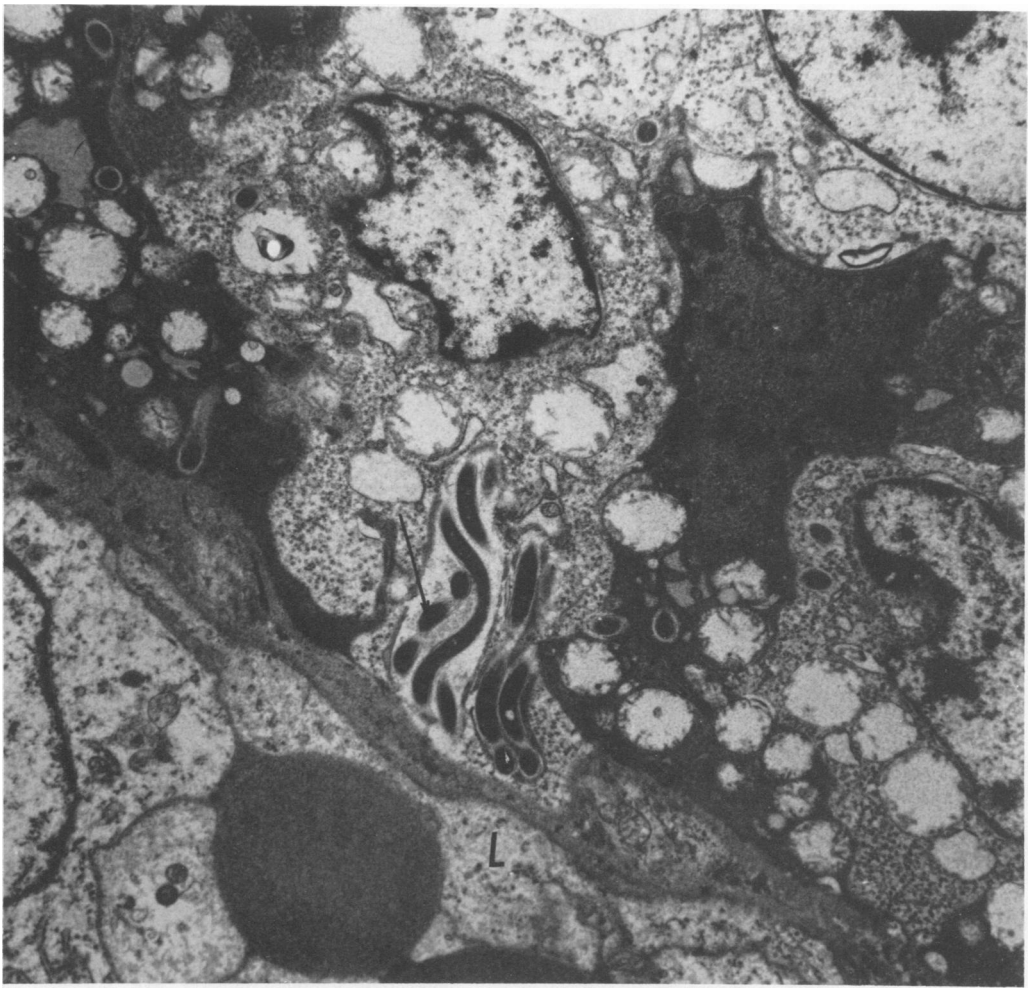

FIG. 9. *Large spirochetes (arrow) at the base of an epithelial cell adjacent to the lamina propria (L).* ×*9,200.*

more likely that some portion of the normal enteric flora may be required for the spirochetes to establish pathogenicity. There is competition between enteric organisms (12, 17), but there are also instances where one type of organism may be dependent on another for some metabolic product or for the creation of a suitable physiological condition (2, 15). Therefore, a type of synergism may exist as previously described (16). Clarification of the interrelationships between various organisms in the enteric flora must await further investigation utilizing animals with a defined microbiota.

The studies described in this paper showed that invasive large spirochetes which had the structural features of *T. hyodysenteriae* were invariably found in large numbers in the mucosa of pigs infected with swine dysentery, but not in normal control animals. Cultures of *T. hyodysenteriae* have been shown to induce swine dysentery in studies conducted in four different countries (1, 5, 7, 14, 22). This evidence is sufficient to lead to the conclusion that *T. hyodysenteriae* is a primary pathogen in the large intestine of swine, but it does not imply that spirochetes are enteric pathogens in other species. It would seem likely that some swine may have nonpathogenic enteric spirochetes with the appearance of *T. hyodysenteriae*, but Akkermans and Pomper (1) were unable to

demonstrate large spirochetes in the colon contents of 168 swine with enteritides other than swine dysentery.

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